Review

Molecular mechanisms underlying the diverse array of petal colors in chrysanthemum flowers

Akemi Ohmiya*

Institute of Vegetable and Floriculture Science, NARO, 2-1 Fujimoto, Tsukuba, Ibaraki 305-0852, Japan

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is one of the most important floricultural crops in the world. Although the origin of modern chrysanthemum cultivars is uncertain, several species belonging to the family Asteraceae are considered to have been integrated during the long history of breeding. The flower color of ancestral species is limited to yellow, pink, and white, and is derived from carotenoids, anthocyanins, and the absence of both pigments, respectively. A wide range of flower colors, including purplish-red, orange, red, and dark red, has been developed by increasing the range of pigment content or the combination of both pigments. Recently, green-flowered cultivars containing chlorophylls in their ray petals have been produced, and have gained popularity. In addition, blue/violet flowers have been developed using a transgenic approach. Flower color is an important trait that influences the commercial value of chrysanthemum cultivars. Understanding the molecular mechanisms that regulate flower pigmentation may provide important implications for the rationale manipulation of flower color. This review describes the pigment composition, genetics, and molecular basis of ray petal color formation in chrysanthemum cultivars.

Key Words: anthocyanin, carotenoid, chlorophyll, chrysanthemum (*Chrysanthemum morifolium* Ramat.), ray petal color.

Introduction

Chrysanthemums were first cultivated in China as herbs around the 15th century BC (Anderson 2006). The plants were introduced to Japan around the 8th century AD, to Europe in the 17th century, and to the United States in the 18th century. The plant was selected and improved in each country to give rise to thousands of cultivars with flowers of various forms and colors. Today, chrysanthemum has become one of the most important floricultural crops in the world.

The ancestry of chrysanthemum cultivars is uncertain. Although they are classified as *Chrysanthemum morifolium* Ramat., in the family Asteraceae, intercrossing has occurred between species, including *C. erubescens*, *C. indicum*, *C. japonense*, *C. ornatum*, *C. sinense*, and *C. makinoi*, which naturally occur in China and Japan (Dowrick 1953). Petal colors of these ancestral species are limited to white, yellow, and pink. During the long history of chrysanthemum breeding, a wide range of petal colors, including purplish-red, red, orange, dark red, and green, has been developed.

Furthermore, application of recent advances in genetic engineering have overcome the limitations of traditional breeding and allowed the addition of novel blue/violet petal color (Noda *et al.* 2013, 2017). These petal colors are formed by the accumulation of a single pigment, a combination of multiple pigments, such as anthocyanins, carotenoids, and chlorophylls, or absence of these pigments (Kishimoto *et al.* 2004, 2007, Nakayama *et al.* 1997, Ohmiya *et al.* 2017).

Flower color is an important trait that determines the commercial value of chrysanthemum cultivars. Hence, improvement of flower coloration has always been a crucial goal for breeders. Understanding the molecular mechanisms that regulate flower pigmentation in chrysanthemums not only provides basic knowledge on pigment science, but may also have important implications for the rationale manipulation of flower color. The chrysanthemum inflorescence consists of central disc florets and marginal ray florets. The color of the disc floret is generally yellow or green, whereas petals of the ray floret (ray petals) exhibit a diverse range of colors that are specific to each cultivar. This review focuses on ray petals and describes the pigment composition, genetics, and molecular basis of ray petal color formation in chrysanthemum cultivars.

1. Yellow

The yellow ray petal color of chrysanthemums is derived from carotenoids (Kishimoto *et al.* 2004). Carotenoids are C40 isoprenoid pigments, widely distributed in nature. Because carotenoids play important roles in photosynthesis (Niyogi 2000), the green tissues of most plants show similar carotenoid profiles: carotenoids that are essential for photosynthesis, such as lutein, β-carotene, violaxanthin, neoxanthin, and zeaxanthin, are invariably found (Goodwin and Britton 1988). In contrast, there is considerable diversity in the carotenoid profiles of different plant species (Ohmiya 2011). In flowering plants, the majority of carotenoids in petals are yellow xanthophylls (oxygenated carotenoids), and they accumulate in the chromoplast (Vishnevetsky *et al.* 1999). The hydroxyl groups of most xanthophylls in petals are esterified with fatty acids via acyl-CoA (Moehs *et al.* 2001, Ohmiya 2013, Yamamizo *et al.* 2010). Recently, an enzyme that catalyzes carotenoid esterification (PYP1: PALE YELLOW PETAL 1) was identified in tomato (*Solanum lycopersicum*) flowers (Ariizumi *et al.* 2014). Loss-of-function of PYP1 causes a drastic decrease in carotenoid content and disruption of normal chromoplast development in petals, suggesting that esterification is an important process for the sequestration of carotenoids into the chromoplast.

1-1. Carotenoid composition in ray petals

Using modern technology, such as nuclear magnetic resonance spectroscopy and fast atom bombardment mass spectrometry, Kishimoto *et al.* (2004) identified 16 carotenoid components contained in the ray petals of chrysanthemums: 14 of which were lutein and lutein derivatives (>90% of the total carotenoid content) (**Table 1**). Lutein is derived from α-carotene, which contains one β-ring and one ε-ring at each end (β, ε-carotenoids), whereas carotenoids derived from β-carotene contain two β-rings (β, β-carotenoids). The proportion of β, ε-carotenoids and β, β-carotenoids differs

Table 1. Carotenoid components in the ray petals of chrysanthemum

(3*S*,5*S*,6*R*,3′*R*,6′*R*)-5,6-dihydro-5,6-dihydroxylutein
(9*Z*,13′*Z*)-lutein 5,6-epoxide
(13*Z*,9′*Z*)-lutein 5,6-epoxide
(9′*Z*,13′*Z*)-lutein 5,6-epoxide
(9*Z*,13*Z*)-lutein 5,6-epoxide
(all-*E*)-lutein 5,6-epoxide
(9*Z*,9′*Z*)-lutein 5,6-epoxide
(9*Z*)-violaxanthin
(8*S*)-lutein 5,8-epoxide
(8*R*)-lutein 5,8-epoxide
(9*Z*-8′*R*)-luteoxanthin
(9′*Z*)-lutein 5,6-epoxide
(9*Z*)-lutein 5,6-epoxide
(all-*E*)-lutein
(9*Z*)-lutein
(9′*Z*)-lutein

among plant species (Ohmiya 2011). For example, among plants belonging to the family Asteraceae, African marigold (*Tagetes erecta*), sunflower (*Helianthus annuus*), calendula (*Calendula officinalis*), and gazania (*Gazania* spp.) have a β, ε-carotenoids-rich composition, whereas zinnia (*Zinnia elegans*) and gerbera (*Gerbera jamesonii*) have a β, β-carotenoid-rich composition (Kishimoto *et al.* 2007).

β-ring and ε-ring formation is catalyzed by lycopene β-cyclase (LCYB) and lycopene ε-cyclase (LCYE), respectively (**Fig. 1**). Several studies have shown that the balance of β, ε- and β, β-carotenoids is profoundly affected by the balance of LCYB and LCYE activity (Chiou *et al.* 2010, Cunningham *et al.* 1996, Cunningham and Gantt 2001, Zhu *et al.* 2003). In chrysanthemums, the level of *LCYE* expression was remarkably higher than that of *LCYB*, suggesting that high LCYE activity determines the flux through the β, ε-carotenoid biosynthesis pathway (Kishimoto and Ohmiya 2006).

Kishimoto *et al.* (2004) found that chrysanthemum ray petals contain various types of isomers of lutein and lutein epoxides, e.g., eight geometrical isomers of lutein-5,6-epoxide (the all-*trans* form, two mono-*cis* forms, and five
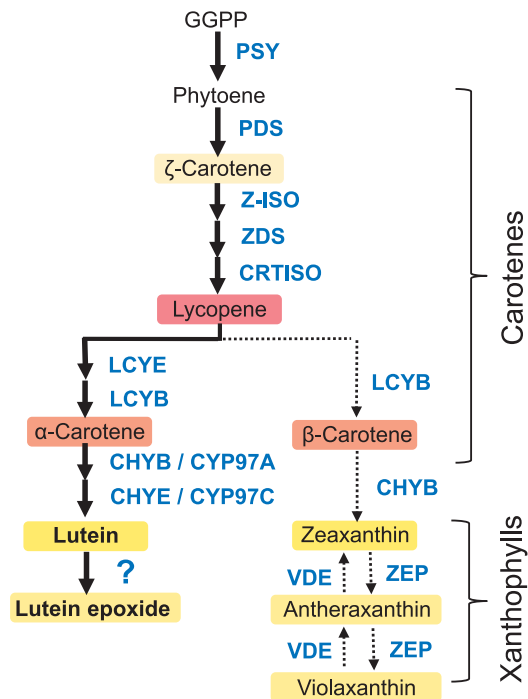

Fig. 1. Schematic representation of the carotenoid biosynthesis pathway in chrysanthemum ray petals. The major carotenoids contained in ray petals of chrysanthemums are lutein and lutein epoxide. Bold and dotted lines indicate the major and minor pathways in ray petals of chrysanthemums, respectively. The background color of each carotenoid represents the typical color of the compound. Enzymes are indicated by blue letters. CHYB, β-ring hydroxylase; CHYE, ε-ring hydroxylase; CRTISO, carotenoid isomerase; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZDS, ξ-carotene desaturase; ZEP, zeaxanthin epoxidase; Z-ISO, 15-*cis*-ξ-CRTISO.

di-*cis* forms; **Table 1**), three geometrical isomers of lutein (the all-*trans* form and two mono-*cis* forms), and two epimeric lutein-5,8-epoxides. Various *cis*-form carotenoids frequently occur in petals (Deli *et al.* 1988, Kull and Pfander 1995, Melendez-Martinez *et al.* 2006, Molnár *et al.* 1986); however, the di-*cis* forms of cyclized carotenoids are particularly rare molecular structures and have only been reported in the petals of several plant species, including rape (*Brassica napus* L.) (Kull and Pfander 1997), calendula (Bakó *et al.* 2002), African marigold (Khachik *et al.* 1999), and pansy (*Viola × wittrockiana* Gams) (Molnár *et al.* 1986). The significance of these unique carotenoid components in petals is still unknown.

2. Pink and purplish-red

Pink and purplish-red ray petal colors are derived from anthocyanins, a subclass of flavonoid compounds. Anthocyanins provide orange, red, purple, and blue colors of many flowers and fruits (Tanaka *et al.* 2008). There are three major classes of anthocyanidins (aglycones of anthocyanins): pelargonidin, cyanidin, and delphinidin (**Fig. 2**). These differ primarily in the number of hydroxyl groups on the B-ring, which affects the color tone of the molecules: the color shifts towards blue as the number of hydroxyl groups
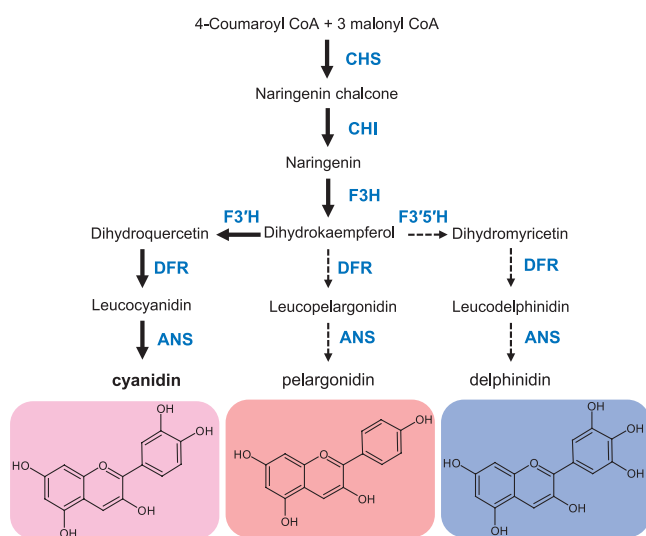

Fig. 2. Schematic representation of the anthocyanidin biosynthesis pathway in chrysanthemum ray petals. Ray petals of chrysanthemums accumulate cyanidin-based anthocyanins. The bold line indicates the pathways leading to the synthesis of cyanidin-based anthocyanins in chrysanthemum ray petals. Pathways leading to the synthesis of pelargonidin- and delphinidin-based anthocyanins (dotted lines) are not detected in chrysanthemum ray petals. Enzymes are indicated by blue letters. The background color of each anthocyanidin structure represents the typical color of the compound; however, the actual color depends on various factors, such as vacuolar pH, metal ions, and the presence of other flavonoid compounds. ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase.

increases. Anthocyanidins are generally modified by sugars and organic acids in a species-specific manner to form anthocyanins. The anthocyanin biosynthesis pathway is positively regulated by transcription factors belonging to three protein families, i.e., R2R3-MYB, basic helix-loop-helix (bHLH), and WD repeat protein (WDR).

2-1. Anthocyanin composition in pink/purplish-red ray petals

The main pigments contained in pink/purplish-red ray petals of chrysanthemums are cyanidin-based anthocyanins: cyanidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside and cyanidin 3-*O*-(3″,6″-*O*-dimalonyl-β-glucopyranoside) (Nakayama *et al.* 1997). The pigmentation of ray petals due to cyanidin-based anthocyanin alone ranges from fairy pinkish to purplish-red, depending on the amount present (from a small to a large amount) (Kawase and Tsukamoto 1976).

2-2. Inheritance of pink/purplish-red ray petal color

Inheritance of pink/purplish-red ray petal color is complicated for the following reasons. Cultivated chrysanthemums are an allohexaploid (2n = 6x = 54), with somatic chromosome numbers varying between 2n = 47 and 63, and have multiple loci (Dowrick 1953). It is difficult to obtain S1 progeny because, like many other plants belong to the family Asteraceae, they are characterized by sporophytic self-incompatibility, which precludes selfing (Stephens *et al.* 1984, Zagorski *et al.* 1983). In addition, chrysanthemums have multiple alleles, which positively or negatively control anthocyanin accumulation (Hattori 1992, Teynor *et al.* 1989). Furthermore, chimerical structure should be considered when analyzing the inheritance of ray petal color. Ray petals consist of two cell layers, L1 (epidermal) and L2 (mesophyll), and anthocyanin pigmentation is restricted in the L1 layer. Because male and female gametes are derived from L2 (Stewart and Dermen 1970), some purplish red-flowered cultivars, which harbor dominant genes for anthocyanin biosynthesis in the L1 but not in the L2 layer, may behave genetically as their white-flowered progenitors.

Induced mutagenesis produced a wide spectrum of ray petal colors in chrysanthemums (Machin and Scope 1978). Mutant lines with either white or purplish-red ray petals could be obtained from pink ray petal cultivars. Ray petals of these mutant lines showed reduced or increased levels of anthocyanins, respectively. In the white-flowered mutant lines, reduced expression of genes encoding either enzymes or positive regulators of anthocyanin biosynthesis were observed (Han *et al.* 2012). In the purplish-red-flowered mutant lines, expression of a R2R3-MYB transcription factor (designated CmMYB1) was markedly reduced (Sung *et al.* 2013). Because the amino acid sequence of CmMYB1 showed high similarity to AtMYB4, which are negative regulators of anthocyanin biosynthesis in Arabidopsis (Jin *et al.* 2000, Matsui *et al.* 2008), the authors suggested that CmMYB1 also acts as a negative regulator of anthocyanin biosynthesis in chrysanthemum ray petals.

2-3. Environmental factors that affect anthocyanin content

Anthocyanin content in ray petals is affected by environmental factors. When chrysanthemums were grown under high temperature conditions (>30°C), there was a significant decrease in the anthocyanin content in the ray petals (Nozaki *et al.* 2006). This phenomenon represents a serious problem for chrysanthemum production during the summer in temperate zone countries, and year-round in tropical zone countries. The response to temperature differed among cultivars: i.e., a marked decrease in anthocyanin content was observed in the flowers of 'Sei-Monako' and 'Sei-Suffle', whereas those of 'Chatoo' were only slightly affected (Nozaki *et al.* 2006). Therefore, the development of cultivars with stable anthocyanin pigmentation under high temperature conditions will be useful for the year-round production of chrysanthemums.

Anthocyanin synthesis is also affected by the light condition. Shading chrysanthemum flowers resulted in a drastic decrease of anthocyanins in ray petals (Hong *et al.* 2015). The expression of genes encoding anthocyanin biosynthesis enzymes, including flavanone 3-hydroxylase (F3H), anthocyanidin synthase (ANS), dihydroflavonol 4-reductase (DFR), and anthocyanindin glycosyltransferase (3GT), and those encoding putative transcriptional activators of anthocyanin biosynthesis (MYB5-1, MYB6, HLH24, CRY1a, COP1, and HY5) were markedly suppressed following shading of the flowers (Hong *et al.* 2016). Although anthocyanin accumulation was most severely impaired when the whole plant body was shaded, a decrease in anthocyanin content was also observed when only the leaves were shaded. Those authors concluded that the leaves perceive the light and transmit the signal to the flowers.

While much is known about how anthocyanin accumulation varies in response to certain environmental stimuli, little is known about how it is regulated at the molecular level. Further study is needed to clarify the mechanism(s) involved.

3. Orange, red, and dark red

The expression of ray petal color in wild chrysanthemum is determined by either anthocyanins or carotenoids. None of them contain both pigments simultaneously. However, crosses between pink/purplish-red and yellow flowers produced a number of chrysanthemum cultivars containing both anthocyanins and carotenoids in their ray petals. Color tone is largely depended on the amount of anthocyanins (Kawase and Tsukamoto 1976). Along with carotenoids, increasing amounts of anthocyanins generate orange, red, and dark-red colors.

3-1. Three ways to develop orange coloration in the family Asteraceae

Analysis of pigment composition in orange ray petals revealed that cultivars belonging to the family Asteraceae can express orange color in three ways (Kishimoto *et al.* 2007).

For example, gerbera and zinnia generate orange petal color in the same way as chrysanthemums, through a combination of yellow carotenoids and red anthocyanins. African marigold and French marigold (*Tagetes patula*) develop an orange color through the accumulation of large amounts of yellow carotenoids, mainly lutein. The orange petal color of calendula, gazania, and African daisy is mainly formed by the accumulation of red carotenoids together with yellow carotenoids. Kishimoto *et al.* (2005) found that red carotenoids accumulated in the orange petals of calendula are lycopenes and carotenes, with a *cis*-configuration. The conversion of *cis*-lycopene to *trans*-lycopene, which is catalyzed by carotenoid isomerase (CRTISO), is an essential step for the formation of yellow xanthophylls (Isaacson *et al.* 2002). Comparison of *CRTISO* sequences expressed in orange and yellow calendula petals revealed several amino acid substitutions and deletions in the deduced amino acid sequences in orange petals (Kishimoto and Ohmiya 2012). Enzyme activity analysis of the recombinant protein showed that the orange petal-specific CRTISO homolog is inactive. This indicates that the orange-flowered calendula is a CRTISO loss-of function mutant that impairs the *cis*-to-*trans* conversion of *cis*-lycopene.

3-2. Possible ability to produce pelargonidin-based anthocyanin

Accumulation of reddish anthocyanin, a pelargonidin-based anthocyanin, is a possible strategy used to produce red petal color. Some plant species, including *Ipomoea*, *Iochroma*, and *Penstemon*, employ the strategy (Des Marais and Rausher 2010, Smith and Rausher 2011, Wessinger and Rausher 2015). However, ray petals of chrysanthemums are unable to synthesize pelargonidin-based anthocyanins. DFR generally exhibits strong substrate preference, and its substrate specificity determines the proportions of pelargonidin, cyanidin, and delphinidin. In *Petunia hybrida* and *Cymbidium hybrida*, the low affinity of DFR to dihydrokaempferol, a pelargonidin precursor (**Fig. 2**), prevents pelargonidin biosynthesis (Forkmann *et al.* 1980, Johnson *et al.* 1999). The results of a feeding experiment showed that chrysanthemum DFR can utilize dihydrokaempferol as a substrate (Schwinn *et al.* 1993), suggesting that the absence of pelargonidin is not caused by the substrate specificity of DFR. Another possible reason for the absence of pelargonidin is the high activity of flavonoid 3′-hydroxylase (F3′H). In chrysanthemum ray petals, dihydrokaempferol is expected to be fully converted by F3′H into dihydroquercetin, and further into cyanidin (**Fig. 2**). Genetic modification or mutation breeding to suppress F3′H activity will enable the production of chrysanthemum flowers that accumulate pelargonidin.

4. Blue

Despite its long history of breeding, chrysanthemum lacks cultivars with blue ray petal color. The majority of blue flowers contain delphinidin-based anthocyanins, which

have three hydroxyl residues (3′-, 4′-, and 5′-positions) on the B-ring (**Fig. 2**). Flavonoid 3′,5′-hydroxylase (F3′5′H) catalyzes hydroxylation at the 3′- and 5′-positions of the B-ring to produce delphinidin-based anthocyanins (**Fig. 2**).

4-1. Transgenic approach for blue coloration

Among members of the family Asteraceae, aster (*Callistephus chinensis*), African daisy, and cineraria (*Pericallis cruenta*) possess F3′5′H activity and accumulate delphinidin-based anthocyanins in their petals (Seitz *et al.* 2006). A comparison of the amino acid sequences of F3′5′H in these plants with those of F3′5′H and F3′H in other plants showed they are more closely related to F3′H than F3′5′H. Phylogenetic analysis suggested that F3′5′H evolved from F3′H before the divergence of angiosperms and gymnosperms, whereas the Asteraceae-specific F3′5′H independently evolved from a F3′H within the family. However, plant species belonging to the genus *Chrysanthemum* in the family Asteraceae cannot synthesize delphinidin-based anthocyanins, because they do not possess F3′5′H activity. Therefore, obtaining blue flowers by cross breeding within the genus is difficult.

Genes encoding F3′5′H have been isolated from various plant species and used as powerful tools to modify flower color (Tanaka 2006, Tanaka and Brugliera 2013). In recent years, transgenic chrysanthemums with blue/violet ray petal color have been produced by expressing a heterologous *F3′5′H* gene under the control of ray petal-specific promoters (Brugliera *et al.* 2013, Noda *et al.* 2013). The majority of cyanidin-based anthocyanins are converted to delphinidin-based anthocyanins, resulting in color of ray petals changing from purplish-red to blue/violet.

Besides the structure of anthocyanidins, several factors affect the development of blue color in flowers, such as vacuolar pH, metal ions, and co-existing colorless compounds (co-pigments) such as flavones and flavonols (Tanaka *et al.* 2008). Noda *et al.* (2017) succeeded in producing blue ray petal color by introducing a gene encoding uridine diphosphate (UDP)-glucose/anthocyanin 3′,5′-glucosyltransferase from butterfly pea (*Clitoria ternatea*), in addition to *F3′5′H* from Canterbury bells (*Campanula medium*). The delphinidins-based anthocyanins produced in the transgenic ray petals were glycosylated at 3′,5′-positions and associated with flavone glucosides, which generates a bluer color.

4-2. Commercialization of blue chrysanthemums

Transgenic carnation (*Dianthus caryophyllus*) and rose (*Rosa hybrida*) with blue/violet flowers expressing *F3′5′H* have already been successfully commercialized, and are available in the cut-flower market worldwide (Chandler and Sanchez 2012, Katsumoto *et al.* 2007, Tanaka and Brugliera 2013). To grow and sell transgenic plants, it is necessary to follow the procedure under the Act on the Conservation and Sustainable Use of Biological Diversity Through Regulations on the Use of Genetically Modified Organisms (Cartagena Act) and obtain government permission (Kikuchi

et al. 2008). It is more difficult to obtain approval for the commercialization of chrysanthemums than for roses and carnations, particularly in Japan, because chrysanthemum cultivars easily hybridize with wild chrysanthemums native to Japan, some of which are classified as endangered species. In addition, the chrysanthemum holds special cultural significance and several symbols and events are held in honor of the chrysanthemum (Anderson 2006).

5. White

White petals contain neither anthocyanins nor carotenoids, whereas most contain flavanones and flavonols, which are subgroups of flavonoids (Chen *et al.* 2012). Although flavanones and flavonols are colorless pigments, the petals express an ivory or cream color when a substantial amount of these pigments are accumulated. There are some cultivars of carnation and snapdragon (*Antirrhinum majus*) with pure white petals due to a complete lack of flavonoid compounds (Onozaki *et al.* 1999, Spribille and Forkmann 1982). However, to date, no chrysanthemum cultivar has been reported that lacks flavonoid compounds in its petals.

5-1. Mechanisms for the lack of anthocyanins

The absence of anthocyanins is generally attributed to a deficiency of the pathway leading to anthocyanin biosynthesis. Such deficiency is generally caused by mutations in biosynthesis enzymes or transcriptional regulators of the pathway. Chen *et al.* (2012) analyzed the expression of anthocyanin biosynthesis genes in white and pink ray petals of chrysanthemum cultivars. They found that the expression of *3GT* and *DFR* in the white petals is extremely low compared with pink petals, suggesting that these enzymes are involved in key regulatory steps in the anthocyanin biosynthesis. However, whether the suppressed expression is caused by changes in the *cis*-regulatory sequence of the enzymes, or by deficiency of transcriptional regulator activity remains to be elucidated.

5-2. Mechanisms for the lack of carotenoids

Inheritance of carotenoid pigmentation in ray petals of chrysanthemums differs from that of anthocyanins: white (absence of carotenoids) is dominant over yellow (presence of carotenoids) (Hattori 1991). In addition, mutations in ray petal color, either spontaneously or induced by mutagens, usually occur from white to yellow, whereas yellow to white mutations rarely occur (Machin and Scope 1978). The existence of a single dominant gene (*I*) that inhibits carotenoid formation and/or accumulation has long been postulated (Boase *et al.* 1997, Hattori 1991, Jordan and Reinmann-Philipp 1983, Stewart and Dermen 1970). However, the precise function of the gene product has remained unknown.

To determine the single dominant gene, Ohmiya *et al.* (2006) performed subtraction screening between white and yellow ray petals and identified a gene specifically expressed in white ray-petals. The deduced amino acid

sequence of the gene has high similarity to carotenoid cleavage dioxygenase (CCD), which oxidatively cleaves carotenoids in a site-specific manner. In Arabidopsis, CCDs fall into nine clades (Tan *et al.* 2003). Five of these, the 9-*cis* epoxycarotenoid dioxygenases AtNCED2, AtNCED3, AtNCED5, AtNCED6, and AtNCED9, are involved in abscisic acid biosynthesis. The remaining four, carotenoid cleavage dioxygenases AtCCD1, AtCCD4, AtCCD7, and AtCCD8, have low sequence homology to the NCEDs. A number of studies have demonstrated that each CCD is involved in various aspects of plant growth and reproduction (Ohmiya 2009). White-petal specific CCD found in chrysanthemums was most closely related to AtCCD4, and was designated as CmCCD4a (Ohmiya *et al.* 2006). Suppression of *CmCCD4a* expression by RNA interference (RNAi) converted the white ray petal color to yellow (Ohmiya *et al.* 2006, 2009), suggesting that white ray petals synthesize carotenoids but CmCCD4a degrades the carotenoids. This hypothesis is supported by the finding that most genes encoding carotenoid biosynthesis enzymes are expressed equally in white and yellow ray petals (Kishimoto and Ohmiya 2006). Subsequently, a ray petal color mutation in chrysanthemums, from white to yellow, was shown to be caused by the loss of the *CmCCD4a* gene (Ohmiya *et al.* 2012, Yoshioka *et al.* 2012). These results indicate that *CmCCD4a* is the single dominant *I* gene that causes inhibition of carotenoid accumulation; however, the results suggest that rather than inhibiting carotenoid formation, the product of this gene catalyzes carotenoid breakdown.

The rate of flower color mutation differs among chrysanthemum cultivars. The white-flowered cultivar 'Jimba', which is the best-selling chrysanthemum cultivar in Japan, rarely or never mutates. A yellow-flowered cultivar with the same growth properties as a white-flowered cultivar would benefit growers, because both forms could be produced under the same conditions. Therefore, much effort has been undertaken to produce a yellow-flowered mutant of 'Jimba'; however, this has yielded limited success. Ohmiya *et al.* (2012) obtained a faint yellow-flowered bud sport of 'Jimba' from a farmer's chrysanthemum field. Following the application of heavy-ion-beam radiation to the bud sport, mutants with pale yellow flowers were obtained. Sub-

Fig. 3. Conversion of chrysanthemum ray petal color from white to yellow by the suppression of *CmCCD4a* expression. NT, non-transgenic 'Jimba'; *ccd4a*, *CmCCD4a* RNAi transformant.

sequently, the mutant was re-irradiated and mutants with much deeper yellow flowers were obtained. Decreased *CmCCD4a* expression correlated well with increased carotenoid content in ray petals of the mutants. *CmCCD4a* comprises a small gene family in the 'Jimba' genome. At least four homologs were expressed in the petals of wild-type 'Jimba' and its bud sports, but only three were expressed in pale-yellow petals, and only one was expressed in yellow petals. Loss of *CmCCD4a* homologs by irradiation was confirmed by genomic PCR analysis. These results suggest that the decreased number of *CmCCD4a* homologs in response to irradiation is responsible for the decrease in expression of *CmCCD4a*, which results in increased carotenoid level.

Specific enzymatic cleavage of carotenoids produces various types of apocarotenoids, some of which provide unique color, flavor, and aroma to the fruits and flowers of many plant species (Ohmiya 2009). An enzyme assay using carotenoid-producing *E. coli* showed that CmCCD4 cleaves β-carotene at positions 9, 10, and 9′,10′ to yield β-ionone, an important aroma constituent in flowers (Huang *et al.* 2009). However, the major carotenoids accumulating in chrysanthemum flowers are lutein and its derivatives (Kishimoto *et al.* 2004), and their cleavage products generated through the action of CmCCD4a remained to be examined.

6. Green

In recent years, there have been increasing numbers of green-flowered chrysanthemum cultivars. The green flower color is derived from chlorophylls, which are magnesium-containing tetrapyrrole macromolecules. Mature leaves generally contain a substantial amount of chlorophylls because they play an essential role in photosynthesis (Niyogi 2000). Petals of many wild flowers contain chlorophylls at an early developmental stage (Pyke and Page 1998). Chlorophyll content decreases as petal development progresses, and mature petals contain only a trace amount of chlorophylls. The absence of chlorophylls is an important trait for wild plants, which enables flowers to be visually distinguished against a background of green leaves when they are ready to offer nectar and pollen to pollinators. In nature, a green-flowered mutant, even if it occurs, may soon disappear because the green flower trait is disadvantageous for reproduction. In contrast, in the case of ornamental plants, green-flowered mutants are preferably selected and developed as cultivars by breeders.

Chlorophyll accumulation is strictly controlled in a tissue-specific manner. The mechanism of chlorophyll accumulation in leaves has been well studied, whereas less attention has been paid to that in flowers. Recently, an expressed sequence tag database of chrysanthemum cultivar 'Sei-Marine' was constructed (Sasaki *et al.* 2017). Based on the database, Ohmiya *et al.* (2017) generated a custom array and performed microarray analyses. Those authors compared the expression levels of chlorophyll-rerated genes among the white petals (absence of chlorophyll), green
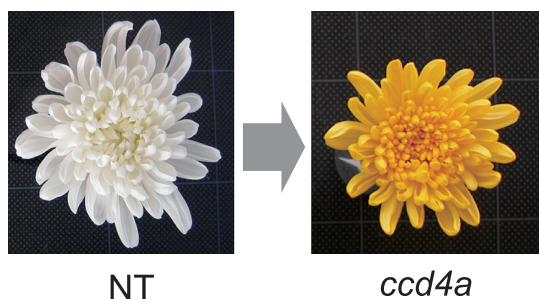
petals (low level of chlorophyll), and leaves (high level of chlorophyll) of chrysanthemum cultivars to identify the mechanisms of chlorophyll accumulation. Their analysis revealed no correlation between the expression levels of chlorophyll catabolic genes and chlorophyll content in petals. However, it is notable that, irrespective of the chlorophyll content in petals, *STAY GREEN* (*SGR*) expression was markedly higher in petals than in leaves. SGR encodes Mg-dechelatase, which catalyzes the conversion of chlorophyll *a* to pheophytin *a*, the first step of chlorophyll degradation (Shimoda *et al.* 2016). There is an increasing evidence that SGR is a key factor controlling chlorophyll degradation (Hörtensteiner 2009, Sakuraba *et al.* 2012). Therefore, the results suggest that SGR maintains high chlorophyll catabolic activity and may contribute to the absence or low level of chlorophylls in petals. In contrast to chlorophyll catabolic genes, the expression of key chlorophyll biosynthesis enzymes, including glutamyl-tRNA reductase, Mg-protoporphyrin IX chelatase, Mg-protoporphyrin IX monomethylester cyclase, and protochlorophyllide oxidoreductase, were well correlated with chlorophyll content, with lower levels observed in white petals compared with green petals, and the highest level found in leaves. Therefore, it seems that the difference in chlorophyll content in the ray petals of chrysanthemum cultivars is determined by the amount of chlorophyll synthesized in the tissue. Similar results have been reported for white- and green-flowered carnation cultivars (Ohmiya *et al.* 2014).

It is reasonable to assume that factors responsible for suppressing chlorophyll biosynthesis or enhancing chlorophyll degradation exist in white petals. Ohmiya *et al.* (2017) identified several candidate transcription factors, whose expression was closely correlated with the chlorophyll level. Functional analysis of these transcription factors will provide a basis for future molecular studies on organ-specific chlorophyll accumulation.

7. Conclusions and perspectives

During the past few decades, the metabolic pathways of anthocyanins, carotenoids, and chlorophylls have been extensively studied, and almost all the enzymes responsible for these pathways have been identified and characterized in model plants (Ohmiya 2013, Tanaka *et al.* 2008, 2011). As described in this article, the genes involved in these metabolic pathways have been cloned in chrysanthemum cultivars based on the nucleotide sequence found in the model plants, and their expression profiles have been analyzed. Findings from these studies have provided us a number of insights into the molecular mechanisms of flower pigmentation. However, there are several issues that remain unsolved, i.e., how the metabolism of pigment compounds is controlled in a tissue-specific manner in chrysanthemums. Positive and/or negative regulators of the metabolic pathways specifically expressed in ray petals may exist. The mechanism regulating pigment accumulation in the specific

cellular organelles also remains to be determined, because pigment content and composition is not controlled solely by the amount synthesized in the cell, but rather the mode of accumulation. Identification of such regulators will provide a deeper understanding of pigment metabolism and accumulation, as well as useful information for the development of molecular markers to accelerate chrysanthemum breeding.

Recently, mRNA sequencing using next-generation sequencing technologies (RNA-seq) has offered novel opportunities for characterizing the transcriptomes of non-model plants (Perez-de-Castro *et al.* 2012). These technologies provide a quick and unbiased approach to the functional annotation of differentially expressed genes, as well as the opportunity to isolate genes of interest, develop molecular markers, and to perform comparative genomic studies. Comparison of transcriptomes among flower pigmentation mutants using RNA-seq will provide us valuable candidates that play important roles in the regulation of metabolism and accumulation of pigment compounds in chrysanthemum flowers. Such knowledge may facilitate further advancement of genetics and breeding of chrysanthemums.

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